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## A COMPARISON OF DNA DAMAGE PROBES IN TWO HMEC LINES WITH X-IRRADIATION

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### ABSTRACT

In this study, we investigated  $\gamma$ H2AX<sup>ser139</sup> and 53BP1<sup>ser25</sup>, DNA damage pathway markers, to observe responses to radiation insult. Two Human Mammary Epithelial Cell (HMEC) lines were utilized to research the role of immortalization in DNA damage marker expression, HMEC HMT-3522 (S1) with an infinite lifespan, and a subtype of HMEC 184 (184V) with a finite lifespan. Cells were irradiated with 50cGy X-rays, fixed with 4% paraformaldehyde after 1 hour repair at 37°C, and processed through immunofluorescence. Cells were visualized with a fluorescent microscope and images were digitally captured using Image-Pro Plus software. The 184V irradiated cells exhibited a more positive punctate response within the nucleus for both DNA damage markers compared to the S1 irradiated cells. The dose and time course will be expanded in future studies to augment the preliminary data from this research. It is important to understand whether the process of transformation to immortalization compromises the DNA damage sensor and repair process proteins of HMECs in order to understand what is "normal" and to evaluate the usefulness of cell lines as experimental models.

### INTRODUCTION

DNA damage from ionizing radiation triggers the mobilization of damage sensor proteins to damage sites resulting in a temporary delay of cell cycle progression and activation of repair machinery [1]. ATM (Ataxia Telangiectasia Mutated) and ATR (Ataxia Telangiectasia and Rad-3-related) proteins are two related kinases central to signaling DNA damage, and recent evidence indicates ATM activation occurs prior to ATR activation following radiation

damage [2]. The published literature of these phenomena has been obtained primarily with human and other mammalian fibroblasts.

A major focus for breast cancer research is to understand the key mechanisms responsible for initiating carcinogenesis. It is the epithelial cell that becomes a cancer cell. Normal epithelial cells have a finite life span and then senesce, and thus are more difficult to study in the laboratory. Evidence exists from studies of human and murine normal mammary glands that multiple epithelial cell-

subtypes exist with distinct patterns of susceptibility to different subtypes of breast cancer [3, 4, 5]. Malignant transformation is a multi-step process in which genetic changes in these different cell subtypes can occur due to exposure to a number of environmental factors, such as viruses, carcinogens, dietary factors, and radiation. Mutations of the TP53 gene are the most frequent genetic lesion in breast cancer and its loss as a result of mutation is thought to be an early step in breast tumorigenesis [6].

Numerous cell culture models derived from Human Mammary Epithelial Cells (HMEC) are available [4, 7, 8]. The role of tumor suppressor proteins, inhibitors of cyclin-dependent kinases, telomerase, and small G proteins have been defined in normal and various stages in the progression to immortalization of HMECs. This paper investigates whether DNA damage foci recruitment after exposure of HMECs to a relatively low dose of X-rays (50cGy) to determine its dependence on immortalization. We have selected two non-malignant HMECs: the 184V non-immortalized HMEC with a finite life span, and the immortalized HMEC HMT-3522 (S1) [9, 10, 11, 12]. Differences in gene expression between these two cell types during normal differentiation into polarized acini have recently been compared to test correlation of marker genes with poor and good prognosis groups among breast cancer patients [13].

Two markers in the ATM DNA damage pathway for radiation-induced effects are compared, histone family 2A member X ( $\gamma$ H2AX), and tumor protein TP53 binding protein 1 (53BP1) [14, 15]. Both proteins are phosphorylated in response to DNA damage [16, 17, 18]. It is possible that the transformation process that immortalizes cells for unlimited growth in culture may affect the DNA damage response pathway. Biological cell models cultured *in vitro* in the laboratory are used to investigate many unknown mechanisms of action underlying molecular responses to stressors in normal human tissues. It is important to understand biological characteristics common to a cell type, as well as differences that may exist in various cell models.

## MATERIALS AND METHODS

### ***Cells and Cell Culture Conditions***

HMEC HMT-3522 (S1) immortalized cells are non-malignant early passage human cells that were made available by Drs. Mina Bissell and Mary Helen Barcellos-Hoff (LBNL). The cells originated from a reduction mammoplasty of a woman with a non-malignant breast lesion and were derived through continuous cell passaging in defined medium [20, 21]. Cells from passages #40-60 were used. These cells were grown at 37°C in a humidified incubator at 5% CO<sub>2</sub> in a chemically-defined medium (DMEM / F12 - H14) composed of DMEM (Dulbecco's Modified Eagle's Medium) plus Ham's F12 (1:1, Invitrogen), 2mM glutamine, 250ng/ml insulin, 5mg/ml sheep prolactin, 10mg/ml transferrin, 10<sup>10</sup> M estradiol (all from Sigma, St. Louis, MO), 10ng/ml EGF, 2.6ng/ml sodium selenite, and 1 $\mu$ M hydrocortisone (from Collaborative Research, MA). Cells were set up in plastic four well LabTek slides or 60mm plastic Falcon petri dishes at 4 x 10<sup>4</sup> cells/well or 1 x 10<sup>5</sup> cells/petri dish, respectively, five days prior to the experiment and were fed every other day.

HMEC 184V is a subtype of the 184 HMEC line that is mortal and non-malignant. These cells were kindly provided by Dr. Martha Stampfer (LBNL), who developed this cell line from a human non-tumorigenic reduction mammoplasty [10, 19]. Cells were grown as described by Stampfer [19] in serum-free MCDB 170+IP Growth Media containing MEBM, (Mammary Epithelial Basal Medium, Clonetics Corporation, San Diego, CA) in a 37°C humidified incubator at 0.2% CO<sub>2</sub>. 184V cells were fed every Monday, Wednesday, and Friday, and transferred at 80% confluency. Cell transfer consisted of 3X rinses of 0.05% trypsin w/EDTA (Invitrogen/GIBCO) of 3, 2, and 1ml, respectively, in 100mm petri dishes. The 184V cells required a 30 second wait between trypsin rinses, with the final trypsinization for 5 minutes at 37°C. Following the final trypsinization, cells were neutralized (with 10ml 1X PBS without Ca or Mg) and spun down in a centrifuge at 1,000 RPM for 5 minutes. Cells were resuspended in growth media, counted, and plated. Cells were plated at 2-4 x 10<sup>4</sup> cells/ml per well into plastic four well LabTek slides three days prior to the experiment.

### ***Irradiation***

Four well LabTek slides and 60mm petri dishes were irradiated one at a time, on a rotating platform, in a 160kVp Pantak X-ray machine, running at 150kVp, 20mA, for a dose of radiation of 50cGy. Dose calibration was done with a NIST-based calibrated Victoreen probe 154. Following irradiation, slides or dishes containing S1 or 184V HMECs were incubated at 37°C at 5.0% and 0.2% CO<sub>2</sub>, respectively, until fixation.

### ***Fixation***

Cells were fixed following irradiation, at 1 hour. Cells were rinsed 2X with PBS and then incubated with Nucleoplasmic Extraction Solution (NES): 0.5% Triton-X, 20mM HEPES (pH 7.9), 50mM NaCl, 3mM MgCl<sub>2</sub>, and 200mM sucrose at RT for 10 minutes. The cells were rinsed 1X with PBS and fixed with 4% paraformaldehyde for 10 mins at RT in a time course after radiation exposure. Cells were rinsed 2X with PBS, and, finally, PBS was added to each area of the slide/dish containing cells, and placed at 4°C overnight.

### ***Immunofluorescence***

Cells fixed with 4% paraformaldehyde were permeabilized in 0.1% NP-40 in PBS for 30 minutes. Cells were blocked with 0.5% casein in PBS for 1 hour, followed by 10% goat serum in PBS for 1 hour. The primary antibodies for  $\gamma$ H2AX<sup>ser139</sup> monoclonal mouse (1:1000 Upstate) or 53BP1<sup>ser25</sup> polyclonal rabbit (1:200 Bethyl Labs) were applied overnight at 4°C in a humidified chamber.

Cells were rinsed 3X20 minutes in PBS, and secondary antibody was applied. Alexa 488 goat anti-mouse (1:300 Invitrogen) or Alexa 594 goat anti-mouse (1:300 Invitrogen) was applied for 1 hour for the S1 or 184V cells, respectively, for  $\gamma$ H2AX<sup>ser139</sup>. Alexa 488 goat anti-rabbit (1:300 Invitrogen) or Alexa 594 goat anti-rabbit (1:300 Invitrogen) was applied for 1 hour for the 184V or S1 cells,

respectively, for 53BP1<sup>ser25</sup>. Alexa 488 produces a green fluorescent signal and Alexa 594 produces a red fluorescent signal.

Cells were rinsed 3 x 15 minutes in PBS, followed by nuclear staining in DAPI (0.25µg/ml) for 5 minutes (nucleus stains blue) covered with vectashield, cover-slipped, and sealed with nail polish.

### Image Acquisition

A Zeiss Axiovert 200M microscope was used to view fluorescent cell signals that were digitally captured with a 40XAPO objective and a QImaging Retiga EX digital CCD camera. Image-Pro Plus software was used to acquire individual 40x images. A minimum of five images from each experimental condition were taken.

### Fluorescence Analysis

The number of fluorescent cells was manually counted for each image field. The fraction of responders in the field was obtained by dividing the number of positive fluorescent nuclei divided by the total number of cells indicated by the blue DAPI stained nuclei. A minimum of 5 images from each sample were analyzed and the data is summarized in Table 1.

HMEC Line	Antibody	Cells Counted					
		Control			1 hr post 50 cGy X-ray		
		+	Total	%	+	Total	%
S1	$\gamma$ H2AX <sup>ser139</sup>	56	257	22.0 ± 8.1%	405	490	83.4 ± 15.8%
	53BP1 <sup>ser25</sup>	2	323	0.7 ± 0.1%	353	524	69.2 ± 12.5%
184V	$\gamma$ H2AX <sup>ser139</sup>	78	329	24.1 ± 7.2%	336	368	91.6 ± 9.7%
	53BP1 <sup>ser25</sup>	230	298	77.9 ± 15.8%	359	368	97.8 ± 2.7%

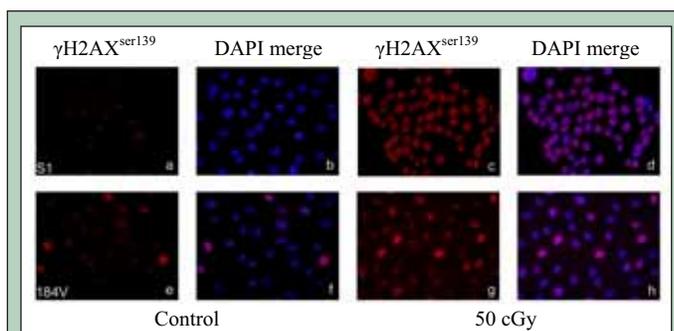
**Table 1.** Number percent of responders for each cell type and marker.

## RESULTS

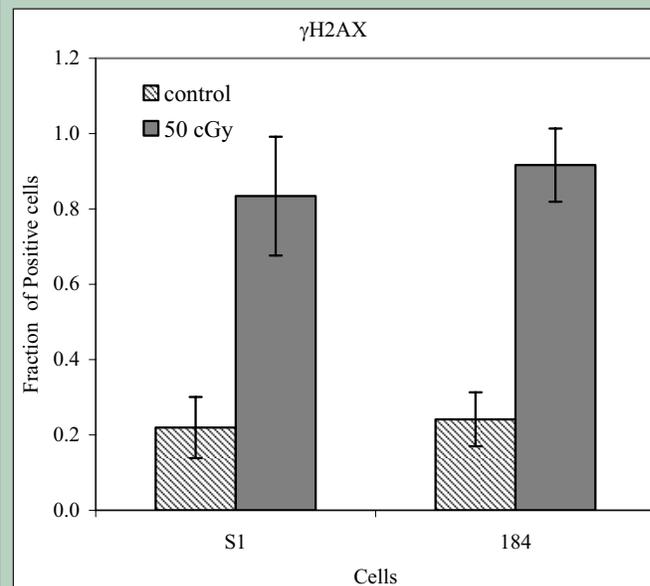
A representative fluorescent image from the 5 fields acquired from each 50cGy irradiated sample after 1 hour of incubation at 37°C, and its respective control for each of the two DNA damage marker proteins with each cell type is depicted in Figure 1A for the  $\gamma$ H2AX<sup>ser139</sup> response, and in Figure 2A for the 53BP1<sup>ser25</sup> response. A histogram representing the quantitative analysis of the fraction of the fluorescence positive cells for the  $\gamma$ H2AX<sup>ser139</sup> response is presented in Figure 1B and for the 53BP1<sup>ser25</sup> in Figure 2B. About 20% of the nonirradiated control samples for the exponentially-growing S1 cells or 184V cells were measured to be  $\gamma$ H2AX<sup>ser139</sup> positive. Although a comparable number of background positive cells were seen for each of the cell types, the nature of the fluorescence was quite different. Most of the signals from the S1 control samples were punctate in nature while some 184V cells showed a distribution of both overall nuclear staining as well as punctate signals. Both cell lines showed increased fluorescent signals one hour after an X-ray dose of 50cGy,

but the fluorescent signals had a different appearance. Most of the positive S1 cells showed global nuclear staining while signals from the 184V cells appeared to contain a distribution of both punctate and overall nuclear staining. Quantitative comparison of the number of positive cells in the DAPI merged H2AX images showed that 91.6 ± 9.7% of the 184V cell nuclei were positive compared to the 83.4 ± 15.8% positive S1 cells. This is illustrated in the histograms in Figure 1B. Error bars show the standard deviation between image fields for the same probe and treatment condition.

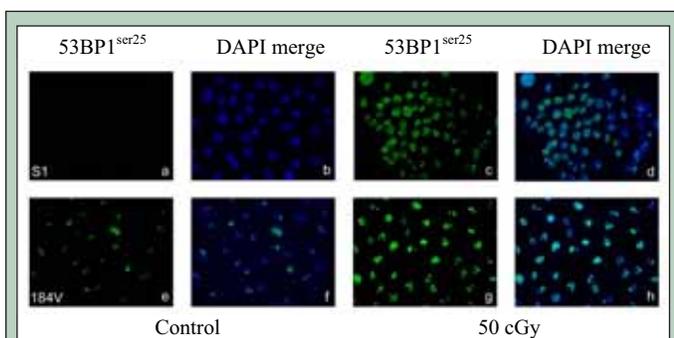
In Figure 2A, the nonirradiated S1 cells showed a very small background signal for 53BP1<sup>ser25</sup> fluorescence. In stark contrast, the 184V cells showed a high background signal of small punctate dots. Both cell types showed increased 53BP1<sup>ser25</sup> fluorescence 1 hour after exposure to 50cGy. Quantitation of the fluorescence signal indicated that 97.8 ± 2.7% of the 184V cells were fluorescence positive, but only 69.2 ± 12.5% of the S1 cells were positive. Results from a student t-test show that the radiation-induced increase in fluorescence in the 184V cells are significantly different (p<0.01) than the S1 cells.



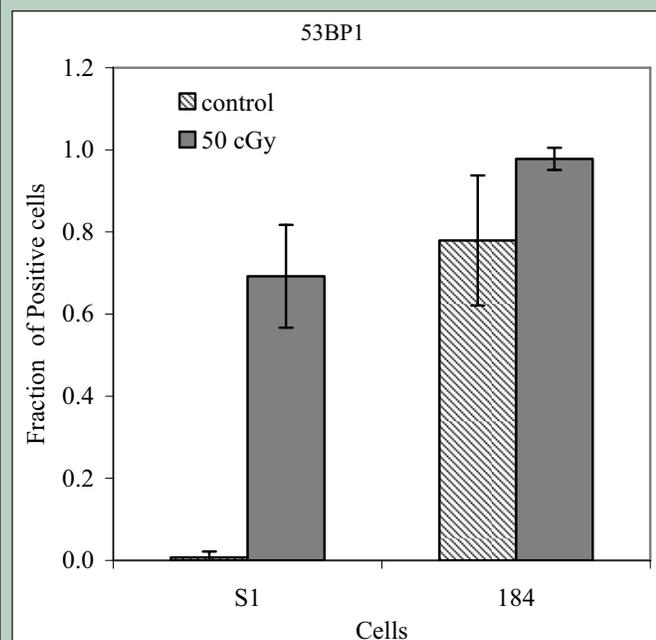
**Figure 1A.**  $\gamma$ H2AX<sup>ser139</sup> response in control (a, b, e, f) and 50cGy (c, d, g, h) images for HMEC S1 (a-d) and HMEC 184V (e-h). Panels a, c, e, g consist solely of the red signal from  $\gamma$ H2AX<sup>ser139</sup> and panels b, d, f, h are color composite images derived from merging the blue DAPI nuclear and the red  $\gamma$ H2AX<sup>ser139</sup> images.



**Figure 1B.** Comparison of the level of positive  $\gamma$ H2AX<sup>ser139</sup> in control or irradiated cell populations, in HMECs S1 or 184V.



**Figure 2A.** 53BP1<sup>ser25</sup> response in control (a, b, e, f) and 50cGy (c, d, g, h) images for HMEC S1 (a-d) and HMEC 184V. Panels a, c, e, g consist solely of the green signal from 53BP1<sup>ser25</sup> and panels b, d, f, h are color composite images derived from merging the blue DAPI nuclear and the green 53BP1<sup>ser25</sup> images.



**Figure 2B.** Comparison of the level of positive 53BP1<sup>ser25</sup> in control or irradiated cell populations of HMECs S1 or 184V.

## DISCUSSION AND CONCLUSIONS

The major observation from the work presented here is that the control background fluorescent signals for the two molecular markers used as indicators for radiation damage are different for the two non-malignant HMECs studied. Furthermore, although it appears that radiation exposure produced a similar level of enhanced  $\gamma$ H2AX<sup>ser139</sup> fluorescent signals in both cell lines, significant differences were observed in both the level and in the pattern of radiation-induced 53BP1<sup>ser25</sup> fluorescence signal. The non-immortalized 184V HMECs showed almost 100% response 1 hour after exposure to 50cGy with each of the two probes,  $\gamma$ H2AX<sup>ser139</sup> and 53BP1<sup>ser25</sup>. The immortalized HMT-3522 S1 HMECs showed approximately 70% 53BP1 response 1 hour after exposure to 50cGy. To our knowledge, this work represents the first documentation of differences in DNA repair markers between two non-malignant HMECs.

Both cell types were exponentially dividing cultures at the time of radiation exposure. One explanation for the observed differences could be due to variable distributions of cells in the cell cycle for each cell type. However, the magnitude of the difference seems unlikely to account for this possibility, but this needs to be ruled out in future studies by examining the cell cycle distribution. The data presented are for a single radiation dose and time point after exposure. It is therefore possible that the time course of the expression of these DNA damage markers is different for each of these cell lines. This would be an interesting observation and future work with a more complete dose response and time course is planned. It is known that multiple genetic changes are required for efficient immortalization of different subtypes of normal human mammary epithelial cells leading to carcinogenesis [4] and that some of these genetic changes can alter the radiation resistance of carcinoma of the breast (22). There are indications that DNA damage markers like  $\gamma$ H2AX<sup>ser139</sup> can be used as therapeutic targets for improving the efficacy of radiation therapy for breast cancer [23] by blocking  $\gamma$ H2AX foci formation or by inhibiting DNA damage repair processes in breast tumor cells. It is important to understand whether the process of transformation to immortalization compromises the DNA damage sensor and repair process proteins of HMECs in order to understand what is “normal” and to evaluate the usefulness of cell lines as experimental models.

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